

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5: C07K 3/14, B01D 57/02		A1	(11) International Publication Number: WO 94/22904
			(43) International Publication Date: 13 October 1994 (13.10.94)
(21) International Application Number: PCT/AU94/00172		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 7 April 1994 (07.04.94)			
(30) Priority Data: PL 8221 7 April 1993 (07.04.93) AU PL 8285 15 April 1993 (15.04.93) AU			
(71) Applicant (for all designated States except US): GRADIPORE LIMITED [AU/AU]; 200 Harris Street, Pyrmont, NSW 2009 (AU).		Published With international search report.	
(72) Inventor; and (75) Inventor/Applicant (for US only): MARGOLIS, Joel [AU/AU]; 23 Valley View Crescent, Greenwich, NSW 2065 (AU).			
(74) Agent: F B RICE & CO; P.O. Box 117, 28A Montague Street, Balmain, NSW 2041 (AU).			
(54) Title: IMPROVEMENTS IN PROTEIN SEPARATION			
(57) Abstract			
<p>A method and apparatus for preparative electrophoresis useful in large scale recovery of macromolecules such as proteins. An electrophoretic potential with an initial polarity is applied across an electrophoretic medium (13) so as to induce at least two species of macromolecules (1, 2) in a mixture (3) with a first electrolyte solution to move into the medium (13) and towards a second electrolyte solution on an opposite side of the medium (13). The at least two species of macromolecules (1, 2) have similar transport characteristics, although at least one (1) of the at least two species of macromolecules (1, 2) move(s) through the medium (13) at a faster rate than the other species of macromolecules (2). The initial polarity is maintained until just before any of the other species of macromolecules (2) have emerged into the second electrolyte solution whereupon the polarity of the electrophoretic potential is reversed while preventing the proportion of the at least one species of macromolecules (1) which has already emerged into the second electrolyte solution from being drawn back into the medium (13). The application and reversal of the electrophoretic potential is repeated until a desired proportion of the at least one species of macromolecules (1) has been transferred to the second electrolyte solution. Preferably, the ratio of times of the initial polarity to the reversed polarity is between 1:2 and 1:4 so that the other species of macromolecules (2) are substantially completely driven back into the first electrolyte solution.</p>			

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

IMPROVEMENTS IN PROTEIN SEPARATIONField of the Invention

The present invention relates to a method of, and apparatus for, preparative electrophoresis and, in particular, to such methods and apparatus useful in large scale recovery of macromolecules such as proteins.

Description of Prior Art

Electrophoretic separation of macromolecules such as proteins, peptides, nucleic acids and other compounds is typically carried out using a medium such as a chemical gel or a membrane. The process involves bringing a first buffer containing a mixture of macromolecule species into contact with the electrophoretic medium whilst an electrophoretic potential is applied along or across the medium so that at least some of the macromolecules are induced to migrate into the medium. The medium typically has a cut-off pore size which is selected so as to allow the entry of molecules having less than a given size. Further, manipulation of the pH of the buffers used in the separation process can contribute to the selection of the molecule species to be separated. The separated molecules are normally caused to emerge from the medium into a second buffer for collection.

A known problem with such electrophoretic separation methods is that molecules larger than the given size will foul the surface of the electrophoretic medium.

This problem has been overcome by a technique of intermittently reversing the polarity of the electrophoretic potential so as to draw the fouling molecules away from the surface of the medium whilst ensuring that there is still a positive movement of the molecules to be separated towards the second buffer. To achieve this, the polarity reversal is typically conducted in short bursts only and the total time of polarity reversal is necessarily a fraction of the time of applying

the "forward" polarity of the electrophoretic potential (see Australian Patent specification 601040).

Typically, the reversal of the polarity is carried out repeatedly at a rate such that the membrane is  
5 constantly being cleared of any surface fouling material. As such, it will be appreciated that the polarity reversal process is analogous to vibrating a sieve. In this model all undersize particles pass through the sieve but  
oversize particles clog it. Vibrating the sieve clears  
10 the oversize particles away from the surface of the sieve and allows more undersize particles to pass through it.

The known electrophoretic separation methods are generally effective for separating macromolecules having different transport characteristics (i.e. size, charge,  
15 shape, isoelectric point) which have actually entered the electrophoretic medium. Where, however, the transport characteristics of two or more of the macromolecules in the medium are similar, it can be difficult to achieve a complete separation of one of the macromolecular species  
20 in the mixture from the others using such conventional methods.

The extent to which two molecule species can be physically separated in an electrophoretic medium will depend not only on their charge characteristics but also  
25 on the mean length of paths that the molecules may follow through the electrophoretic medium. Even for a very long mean path length there may not be complete separation of the molecule species due to molecules of the faster moving species being trapped in the medium and contaminating the  
30 slower moving molecular species. Even if merely lengthening the medium would achieve complete separation of two species that have similar transport characteristics, the use of such an elongate electrophoretic medium would be inconvenient and there may  
35 be practical problems to overcome, such as heat

dissipation.

In contrast, the present method and apparatus utilises the observed differential in the rate of progress of components using a selected electrophoretic medium and/or pH of the buffer so that different species of macromolecules can be conveniently and generally completely separated from one another despite similarities in their transport characteristics.

Summary of the Invention

The present invention provides a method for the electrophoretic separation of at least one species of macromolecules from a mixture with at least one other species of macromolecules by causing the electrophoretic migration of the at least one species of macromolecules from a first electrolyte solution to a second electrolyte solution through an electrophoretic medium separating those solutions, the medium having paths therethrough with a cross-sectional dimension sufficient to allow at least the at least two species of macromolecules in the mixture to pass therethrough and with a mean path length for the movement of molecules through the medium which is very large relative to the effective size of at least one of the species of macromolecules in the mixture, the method comprising:

applying an electrophoretic potential across the electrophoretic medium with an initial polarity to drive the macromolecules of the mixture into the medium until a proportion of the at least one species of macromolecules emerges from the medium into the second electrolyte solution and the other species of macromolecules have penetrated a substantial distance through the medium but before the other specie(s) of macromolecules have so emerged;

reversing the polarity of the electrophoretic potential so as to drive the macromolecules which are in

the medium back towards the first electrolyte solution while substantially preventing the proportion of the at least one species of macromolecules that has emerged into the second electrolyte solution from being drawn back into the medium; and

repeating the application and reversal of the electrophoretic potential until a desired proportion of the at least one species of macromolecules has been transferred to the second electrolyte solution.

Preferably, the step of reversing the polarity of the electrophoretic potential is continued for a sufficient time to allow the other specie(s) of macromolecules to be substantially completely driven back into the first electrolyte solution before re-establishing the initial polarity of the electrophoretic potential and repeating the cycle of application and reversal of the polarity of the electrophoretic potential.

Preferably, the proportion of the one species of macromolecules that has emerged into the second electrolyte solution is substantially prevented from being drawn back into the medium by selectively replacing the second electrolyte solution with fresh second electrolyte solution during the step of reversing the polarity of the electrophoretic potential.

Preferably, the proportion of the at least one species of macromolecules that has emerged into the second electrolyte solution is transported to a first downstream reservoir during the step of applying the initial polarity of the electrophoretic potential and wherein, during the step of reversing the polarity of the electrophoretic potential, a second downstream reservoir supplies the fresh second electrolyte solution.

The reverse polarity of the electrophoretic potential is preferably maintained for a time that is at least equal to no more than the time that the initial polarity is

maintained. More preferably the ratio of the times of the initial and reverse polarities is from 1:2 to 1:4.

The electrolyte solutions are preferably formulated buffers to keep the electrophoretic medium at a desired  
5 pH. Alternatively, this may be achieved by the incorporation of immobilised amphoteric buffering compounds into the electrophoretic medium.

The length of the mean path for the molecules through the electrophoretic medium is preferably at least 10,000  
10 times, and more preferably at least 20,000 times, as long as the effective size of the one species of macromolecules. The effective size of the macromolecules is the maximum size measured in the direction of movement of the macromolecule through the electrophoretic medium  
15 under the applied potential serving to draw the macromolecules through the medium. Typically, the length of the mean path, which will of course normally be somewhat longer than the thickness of the electrophoretic medium, will be extremely large relative to the effective  
20 size of the macromolecules. The measurement of the effective size of the molecules or of the mean path length are thus not normally matters of critical importance. It would require the electrophoretic medium to be a membrane of extreme thinness before the matter became critical.

25 The present invention also provides apparatus for the electrophoretic separation of at least one species of macromolecules from a mixture containing at least one other species of macromolecules, comprising:

a first electrolyte solution compartment to contain  
30 the mixture and a first electrolyte solution;

a second electrolyte solution compartment to contain an electrolyte solution for collecting the one species of macromolecules;

an electrophoretic medium separating the first and  
35 second compartments, the medium having pores of a

- 6 -

cross-sectional dimension sufficient to allow the macromolecules to pass therethrough and having a mean path length for molecules passing through the medium which is very large relative to the effective size of the  
5 macromolecules of at least one of the species of macromolecules in the mixture;

first and second electrodes on opposite sides of the medium for applying an electrophoretic potential across the medium with an initial polarity to induce the  
10 macromolecules to move from the first electrolyte solution compartment into the medium and towards the second electrolyte solution compartment;

control means to reverse the polarity of the electrophoretic potential at a first predetermined time  
15 after applying the initial polarity of the electrophoretic potential, which first predetermined time is after a proportion of the at least one species of macromolecules have emerged into the second electrolyte solution compartment and before any of the other species of  
20 macromolecules have so emerged; to re-establish the initial polarity of the electrophoretic potential after a second predetermined time which is sufficient to cause the macromolecules which are in the medium to migrate back towards the first electrolyte solution compartment a  
25 predetermined distance; and to repeat the application and reversal of the polarity of the electrophoretic potential for a predetermined period until a desired proportion of the one species of macromolecules has been transferred from the mixture to the electrolyte solution in the second  
30 compartment.

Preferably, the second predetermined time is sufficient to cause substantially all of the macromolecules which are in the membrane to migrate back into the first electrolyte solution compartment.  
35 Preferably, the second predetermined time is at least



equal to the first predetermined time. More preferably, the ratio of the first predetermined time to the second predetermined time is from 1:2 to 1:4.

Preferably, the control means also causes the second  
5 electrolyte solution compartment to fluidly communicate with a first downstream reservoir during the step of applying the initial polarity of the electrophoretic potential so as to collect the electrolyte solution containing the separated macromolecules of the one species  
10 of macromolecules, and automatically disconnects the second compartment from the first downstream reservoir and connects it to a second downstream reservoir which supplies a fresh electrolyte solution to the second compartment during the step of reversing the polarity of  
15 the electrophoretic potential.

Preferably, the predetermined period is sufficient to remove a significant proportion, preferably at least 40% and more preferably at least 50% of the at least one species of macromolecules from the mixture and concentrate  
20 same in the electrolyte solution in the second compartment and first downstream reservoir.

In one preferred embodiment, the apparatus includes a plurality of mediums arranged in a series having decreasing pore size, each membrane to be used to separate  
25 a respective species of macromolecules from the mixture.

In the same way that the prior art arrangement involving reversal of polarity could be likened to vibrating a sieve, the process according to the present invention can be likened to a fun-run. Using the fun-run  
30 analogy, the present invention is like separating male runners from female runners with the knowledge that, on average, male runners are faster than female runners. A large group containing a mixture of male and female runners is assembled at the start line with the race  
35 course (i.e. from start to finish) being analogous to the

electrophoretic medium of the present invention. The race is then started with male and female runners racing towards the finish line. It can be predicted that a certain number of male runners will finish the fun-run before any of the female runners. The fun-run is purposely stopped just prior to any female runners crossing the finish line. All of the runners which have not yet crossed the finish line would then be sent back to start another race. The fun-run races can then be repeated until a desired proportion of the male runners have been separated from the female runners.

#### Brief Description of the Drawings

A preferred form of the present invention will be described by way of example with reference to the accompanying drawings, wherein:

Figures 1 and 2 are schematic views of the electrophoretic separation process of the present invention at two separate stages of the process;

Figure 3 is a schematic view of the electrophoretic separation apparatus of the present invention.

#### Best Method of Carrying out the Invention

The apparatus 10 shown in Figure 3 includes an upstream buffer compartment 11 and a downstream buffer compartment 12 separated by an electrophoretic membrane 13. The compartments 11, 12 have outer walls 14 which are permeable to ion solutions but not to macromolecules such as protein.

Electrodes 15, 16 are provided outside the upstream and downstream compartments 11, 12 so as to be on opposite sides of the membrane 13. The electrodes 15, 16 are used to apply an electrophoretic potential across the membrane 13.

The first compartment 11 communicates via tubes 24, 25 with an upstream reservoir 17 so that commercial quantities of a mixture containing various

species of macromolecules can be circulated as a first buffer stream past the membrane 13. The mixture 3 contains a predetermined species 1 of macromolecules which is desired to be separated from other specie(s) 2 of macromolecules having similar transport characteristics which is/are also contained in the mixture 3.

The downstream compartment 12 selectively communicates via tubes 20, 21 with either a first downstream reservoir 18 or a second downstream reservoir 19 which are arranged in parallel. The first downstream reservoir 18 is used to collect buffer containing the one species of macromolecules which has been separated from the mixture, and the second downstream reservoir 19 contains a fresh aliquot of buffer solution.

The downstream reservoirs 18, 19 are connected to the tubes 20, 21 by a pair of control valves 22, 23 so that a control device (not shown) can fluidly connect one or the other of the reservoirs 18, 19 to the downstream compartment 12.

In use, the mixture 3 containing the two or more species of macromolecules 12, including a particular species of macromolecules 1 which is to be separated from the mixture 3, is contained in the upstream reservoir 17 and is circulated as a first buffer stream through the tube 24, the upstream compartment 11 and back to the reservoir 17 through the tube 25.

The membrane 13 is selected having a predetermined pore size so that the species of macromolecules 1 which is to be separated from the mixture 3 will progress through the membrane 13 at a faster rate than any of the other species of macromolecules 2 in the mixture. A suitable electrophoretic potential is applied across the membrane 13 by means of the electrodes 15, 16 with an initial polarity for inducing the macromolecules 1, 2 to migrate from the mixture in the upstream compartment 11

into the membrane 13 and towards the downstream compartment 12.

The initial polarity of electrophoretic potential is maintained whilst the species of macromolecules 1 to be separated moves through the membrane 13 and emerges into the downstream compartment 12 and for so long as substantially none of the other species of macromolecules 2 have emerged into the downstream compartment 12. In this phase of the process, the first downstream reservoir 18 is caused to be connected to the downstream compartment 12 so that the separated species of macromolecules 1 can be collected.

At a first predetermined time when the other species of macromolecules 2 are about to emerge from the membrane 13 into the downstream compartment 12, the polarity of the electrophoretic potential is reversed so as to induce all of the macromolecules 1, 2 within the membrane 13 to migrate back towards the upstream compartment 11. The reversed polarity of the potential is maintained for a second predetermined time until substantially all of the macromolecules 1, 2 have emerged from the membrane 13 back into the upstream compartment 12 whereupon the initial polarity is re-established to start a new cycle.

During the reversed polarity phase of the process, the second downstream reservoir 19 is caused to be connected to the downstream compartment 12 so as to supply a fresh aliquot of buffer solution into the downstream buffer compartment 12. In this way, the proportion of the species of macromolecules which has already emerged from the membrane 13 into the downstream solvent stream is moved out of the downstream compartment 12 and replaced with the fresh aliquot of buffer solution. This generally avoids any of the one species of macromolecules which have already been separated from being induced back into the

membrane 13.

The above process can be repeated on the mixture using a series of membranes 13 having different pore sizes and/or by changing the pH of the buffer so as to

5 successively separate different species of macromolecules contained in the mixture. For example, the membranes 13 for each successive stage of the separation process may have decreasing pore size to create a discontinuous pore gradient.

10 It will be appreciated that the selection of the membrane, the buffer, and the above first and second predetermined times for applying and reversing the electrophoretic potential must first be determined by trials using, for example, coloured markers. Once the  
15 most appropriate membrane and buffer solution for separating a particular species of macromolecules from a particular mixture have been selected, and the times for applying the electrophoretic potential and reversing the polarity of the potential respectively have been  
20 determined for the selected membrane and buffer, the process can be easily automated using appropriate control devices.

Further, the cycle of applying and reversing the electrophoretic potential in accordance with the present  
25 invention will also serve to "flush out" any macromolecules which foul the surface of the membrane 13.

#### Example

The apparatus described in Figure 3 was used for the separation of stained Lysozyme monomer (MW 14kD) from  
30 dimer (28kD) and higher polymers from a mixture thereof. The buffer used in the first and second buffer compartments was Tris-Borate-EDTA at a concentration of 50mM giving a buffered pH of 8.3.

The electrophoretic media comprised a stack of four  
35 membranes each having a molecular weight exclusion of

- 12 -

approximately 50kD. The total thickness of 0.4mm. The electrophoretic current was 180V, 0.4A with a brief reverse pulse periodically to reduce surface fouling of the membranes.

5       The apparatus was operated with an initial forward polarity for three minutes followed by a reverse polarity for six minutes. It was found that substantially pure monomer was accumulated in the second buffer. When the initial forward polarity was increased to five minutes the  
10 second buffer solution was found to contain dimer.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the  
15 invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

## CLAIMS:

1. A method for the electrophoretic separation of at least one species of macromolecules from a mixture with at least one other species of macromolecules by causing the  
5 electrophoretic migration of the at least one species of macromolecules from a first electrolyte solution to a second electrolyte solution through an electrophoretic medium separating those electrolyte solutions, the medium having paths therethrough with a cross-sectional dimension  
10 sufficient to allow at least the at least two species of macromolecules in the mixture to pass therethrough and with a mean path length for the movement of molecules through the medium which is very large relative to the effective size of at least one of the species of  
15 macromolecules in the mixture, the method comprising:  
applying an electrophoretic potential across the electrophoretic medium with an initial polarity to drive the macromolecules of the mixture into the medium until a proportion of the at least one species of macromolecules  
20 emerges from the medium into the second electrolyte solution and the other species of macromolecules have penetrated a substantial distance through the medium but before the other specie(s) of macromolecules have so emerged;  
25 reversing the polarity of the electrophoretic potential so as to drive the macromolecules which are in the medium back towards the first electrolyte solution while substantially preventing the proportion of the one species of macromolecules that has emerged into the second  
30 electrolyte solution from being drawn back into the medium; and  
repeating the application and reversal of the electrophoretic potential until a desired proportion of the at least one species of macromolecules has been  
35 transferred to the second electrolyte solution.

2. The method of claim 1 wherein the step of reversing the polarity of the electrophoretic potential is continued for a sufficient time to allow the other specie(s) of macromolecules to be substantially completely driven back  
5 into the first electrolyte solution before re-establishing the initial polarity of the electrophoretic potential and repeating the cycle of application and reversal of the polarity of the electrophoretic potential.
3. The method of claim 2 wherein the proportion of the  
10 one species of macromolecules that has emerged into the electrolyte solution is substantially prevented from being drawn back into the medium by selectively replacing the second electrolyte solution with fresh second electrolyte solution before the step of reversing the polarity of the  
15 electrophoretic potential.
4. The method of claim 3 wherein the proportion of the one species of macromolecules that has emerged into the second electrolyte solution is transported to a first downstream reservoir during the step of applying the  
20 initial polarity of the electrophoretic potential and wherein, during the step of reversing the polarity of the electrophoretic potential, a second downstream reservoir supplies the fresh second electrolyte solution.
5. The method of claim 1 wherein the cycle of applying  
25 and reversing the electrophoretic potential is repeated until a significant proportion of the one species of macromolecules has been removed from the first electrolyte solution and collected in the second electrolyte solution.
6. The method of claim 1 wherein the mean path length of  
30 the electrophoretic medium is at least 10,000 times as long as the effective size of at least one of the species of macromolecules in the mixture.
7. The method of claim 1 in which the electrolyte solutions are pH buffers.
- 35 8. The method of claim 1 in which the ratio of the time



of the initial and the reverse polarities is from 1:2 to 1:4.

9. An apparatus for the electrophoretic separation of at least one species of macromolecules from a mixture  
5 containing at least one other species of macromolecules, comprising:

a first electrolyte solution compartment to contain the mixture and a first electrolyte solution;

a second electrolyte solution compartment to contain  
10 an electrolyte solution for collecting the at least one species of macromolecules;

an electrophoretic medium separating the first and second compartments, the medium having paths with a cross-sectional dimension sufficient to allow the  
15 macromolecules to pass therethrough and with a mean path length for molecules passing through the medium which is very large relative to the effective size of the macromolecules of at least one of the species of macromolecules in the mixture;

20 first and second electrodes on opposite sides of the medium for applying an electrophoretic potential across the medium with an initial polarity to induce the macromolecules to move from the first compartment into the medium and towards the second compartment;

25 control means to reverse the polarity of the electrophoretic potential at a first predetermined time after applying the initial polarity of the electrophoretic potential, which first predetermined time is after a proportion of the at least one species of macromolecules  
30 have emerged into the second compartment and before any macromolecules of the other species of macromolecules have so emerged; to re-establish the initial polarity of the electrophoretic potential after a second predetermined time which is sufficient to cause the macromolecules which  
35 are in the membrane to migrate back towards the first

- compartment a predetermined distance; and to repeat the application and reversal of the polarity of the electrophoretic potential for a predetermined period until a desired proportion of the at least one species of
- 5 macromolecules has been transferred from the mixture to the electrolyte solution in the second compartment.
10. The apparatus of claim 9 wherein the second predetermined time is sufficient to cause generally all of the macromolecules which are in the membrane to migrate
- 10 back into the first electrolyte solution compartment.
11. The apparatus of claim 9 wherein the control means also causes the second electrolyte solution compartment to fluidly communicate with a first downstream reservoir during the step of applying the initial polarity of the
- 15 electrophoretic potential so as to collect the electrolyte solution containing the separated macromolecules of the at least one species of macromolecules, and disconnects the second compartment from the first downstream reservoir and connects it to a second downstream reservoir which
- 20 supplies fresh electrolyte solution to the second compartment during the step of reversing the polarity of the electrophoretic potential.
12. The apparatus of claim 9 including a plurality of mediums arranged in a series having decreasing pore size,
- 25 each medium to be used to separate a respective species of macromolecules from the mixture.

1/2

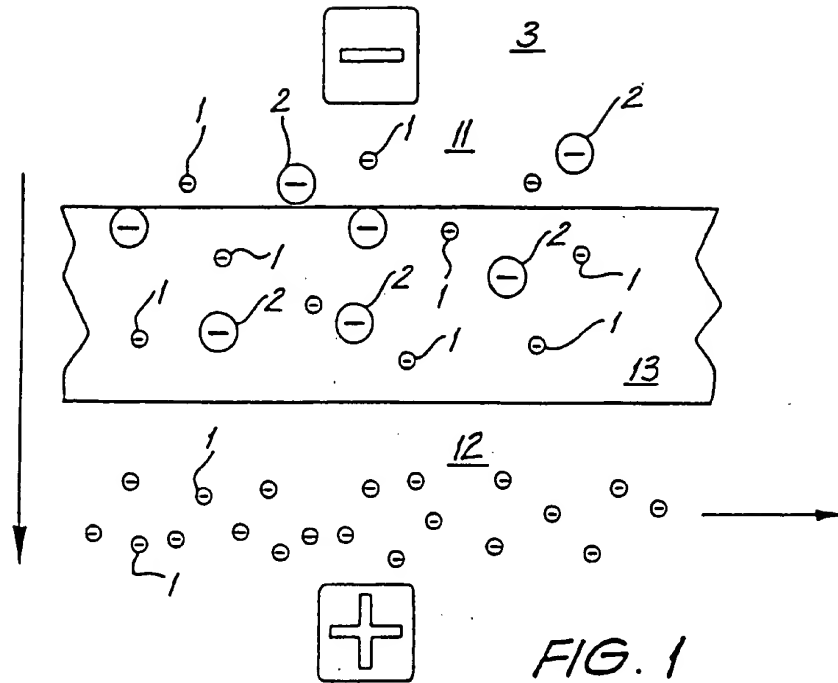


FIG. 1

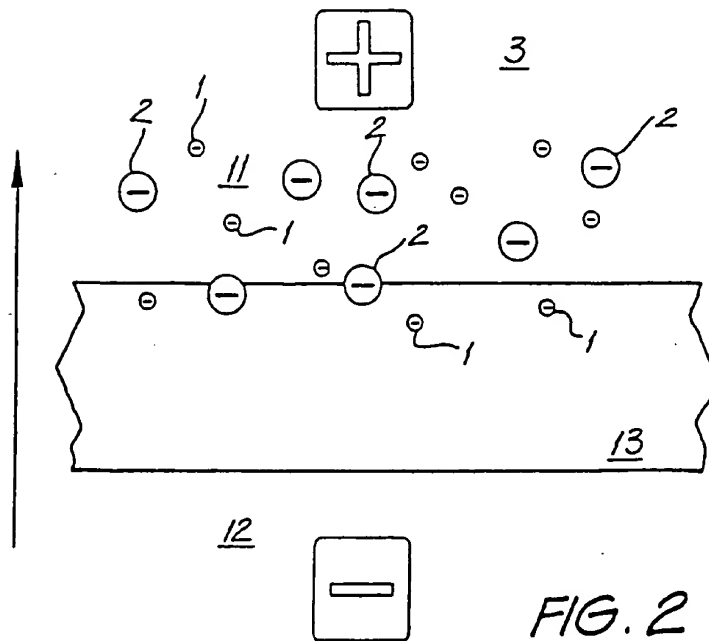


FIG. 2

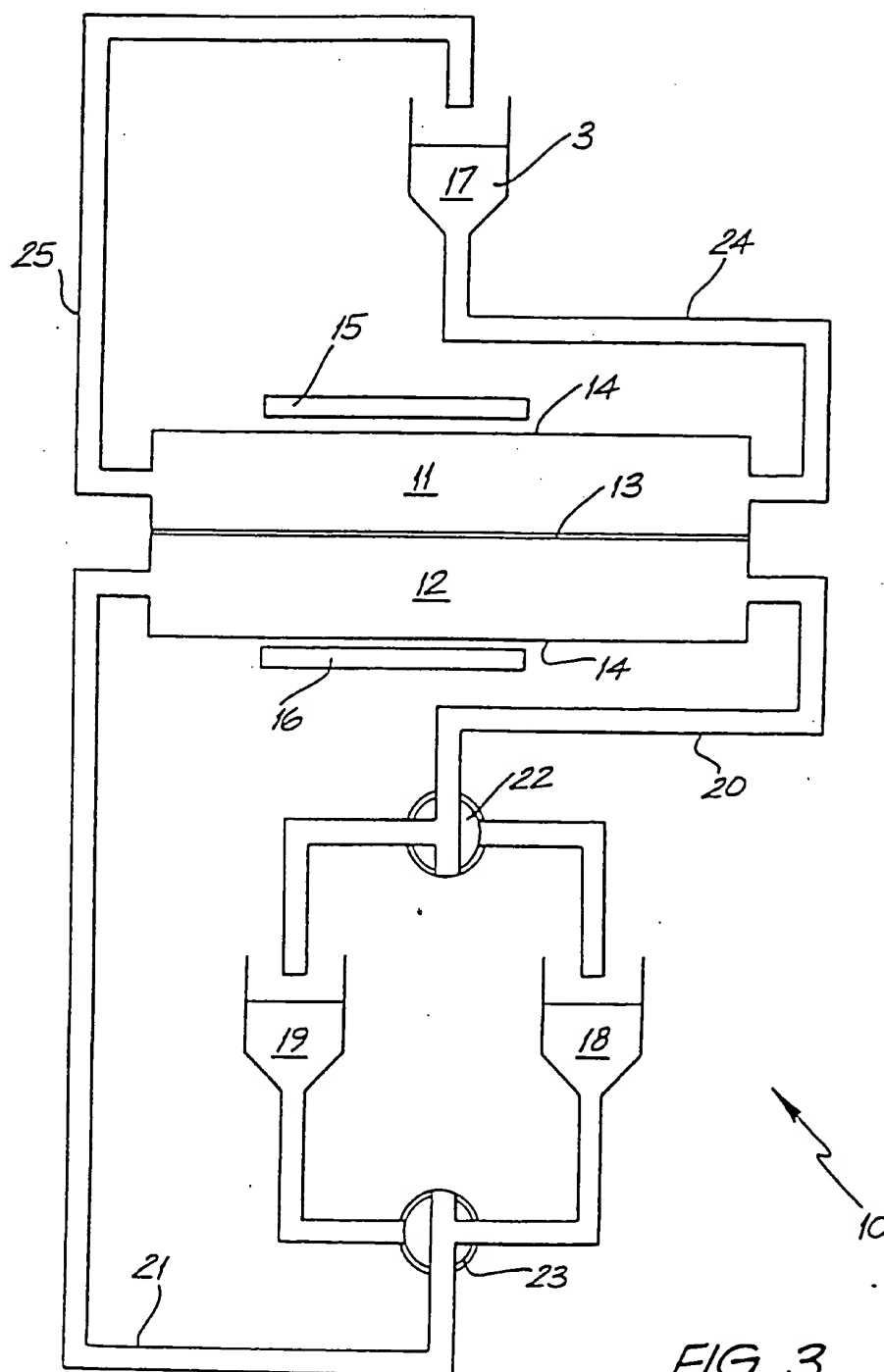



FIG. 3

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 94/00172

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <sup>5</sup> C07K 3/14, B01D 057/02 According to International Patent Classification (IPC) or to both national classification and IPC																						
<b>B. FIELDS SEARCHED</b>  Minimum documentation searched (classification system followed by classification symbols) IPC C07K 3/14, B01D 057/02  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU : IPC as above  Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) FILE WPAT (DERWENT DATABASE) KEYWORDS: ELECTROPHORESIS, ELECTROPHORETIC SEPERAT., REVERS., POTENTIAL FILE CASM (CHEMICAL ABSTRACTS) KEYWORDS: as above																						
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.																				
X, Y	AU, B, 65402/86 (591399) (WASHINGTON UNIVERSITY) 24 April 1987 (24.04.87) whole document	1, 12																				
X, Y	Preparative Biochemistry, Vol 14 No 3 1984 pp 205-221 Abrahamovitz A.S. et al "Recovery of native proteins from preparative electrophoresis gel slices by reverse polarity elution" whole document																					
Y	US, A, 5133844 (STEVENS) 28 July 1992 (28.07.92) whole document  (continued)	1-12																				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.																						
* Special categories of cited documents : <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier document but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&amp;"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 1 June 1994 (01.06.94)		Date of mailing of the international search report 6 June 1994 (06.06.94)																				
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  G.R. PETERS Telephone No. (06) 2832244																				

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU 94/00172

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	AU.B, 16280/88 (601040) (GRADIPORE LIMITED) 2 November 1988 (02.11.88) whole document	1-12
Y	WO,A, 87/00635 (HOEFER SCIENTIFIC INSTRUMENTS) 29 January 1987 (29.01.87) whole document	1-12
Y	EP,A, 396053 (ISCO, INC) 7 November 1990 (07.11.90) whole document	1-12
A	US,A, 3720593 (EVA Th) 13 March 1973 (13.03.73)	1-12

**INTERNATIONAL SEARCH REPORT**

Information on patent family memb

International application No.

PCT/AU 94/00172

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	65402/86	CA	1292439	EP	239624	US	4737251
		WO	8701955	US	5167790		
AU	16280/88	EP	352286	US	5039386	WO	8807406
		CA	2068474	EP	515231	JP	5194837
WO	87/00635	EP	231239	GB	8518030	US	5176805
		GB	8602089				
EP	396053	CA	2016107	JP	3041356	US	5135628
END OF ANNEX							